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RNA editing in wheat mitochondria proceeds by a deamination mechanism

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Abstract Most if not all mitochondrial messenger RNAs from seed plants undergo a post-transcriptional modification (RNA editing) involving the conversion of some cytidine residues to uridine. Using a molecular hybridization approach, an in vitro RNA editing system, able to faithfully reproduce the in vivo observed C to U changes of subunit 9 (*atp9*) of wheat mitochondrial ATP synthase mRNA, has been described [Araya et al. (1992) Proc. Natl. Acad. Sci. USA 89, 1040–1044]. In this work we extend these studies to better understand the biochemical mechanism of this process. RNA editing was analysed by P1 nuclease digestion of the reaction product followed by thin layer chromatography. Experiments performed with unedited [^{3}H]RNA labelled on the base and with unedited [^{32}P]RNA labelled at the α-phosphate of cytidine residues, indicate that plant mitochondrial RNA editing operates through a deamination mechanism.

Key words: RNA editing; ATPase subunit 9; Plant mitochondrion; *Triticum aestivum*

1. Introduction

There is increasing evidence that in many organisms a modification of the genetic message is produced by RNA editing. The role played by this post-transcriptional modification remains elusive but there is good evidence that it may be crucial in the acquisition of proteins with particular functions [1,2].

The functional consequences of RNA editing in plant mitochondria are less understood [3–5]. Recent results suggest that mRNA editing plays an important role for the production of functional proteins [6]. RNA editing has been also described in viruses, mammalian, protozoa, fungi and plants. All these organisms do not share the same biochemical mechanism to fulfill this function. Thus, depending on the organism, RNA editing can operate through different mechanisms such as transcriptase stuttering, nucleotide additions and excision or base modification (for a review see [7]).

RNA editing is widely found in mitochondria of most land plants [8]. Contrary to the mammalian system where RNA editing seems tissue-specific and is restricted to a very limited number of transcripts, RNA editing in plant mitochondria is produced in most plant tissues and involves almost all the protein coding transcripts and some structural RNAs ([9] and references therein). RNA editing in plant organelles is characterized by C-to-U conversion of some residues in the transcript. It is an important process during gene expression in plant mitochondria since the organelle encoded proteins are issued from edited mRNAs [10]. RNA editing occurs also in some chloroplast transcripts although at a lesser extent than in mitochondria [11].

The mechanism and the biochemical characterization of the plant mitochondrial mRNA editing system are not well documented. In this work we report results showing that a deamination mechanism is operating in plant mitochondria RNA editing.

2. Materials and methods

2.1. RNA synthesis

Plasmid pG300 containing the complete coding region of subunit 9 of mitochondrial ATP synthase gene (*atp9*), pHNED3′-S carrying an insert of 66 nucleotides bearing codons 61–75 of *atp9* [9] and pNADC2 containing the sequence from −8 to 17 of *nad3* [12] were used for in vitro synthesis of unedited RNA substrates. In vitro transcription was catalyzed by T7 RNA polymerase. The reaction mixture contained 40 μM [α-^{32}P]CTP (150 Ci/mmol) or 100 μM [5-^{3}H]CTP (25 Ci/mmol) and 400 μM of the other three ribonucleosides triphosphates. The in vitro synthesized RNAs were separated from NTP precursors by chromatography on Sep-Pak columns (Waters). The substrates used for this work are shown in Fig. 1.

Mitochondrial extract from wheat embryos was prepared as described [13].

2.2. Editing assay

Labelled substrate was incubated with protein fractions as described [13]. P1 nuclease digestion of *atp9* RNA was performed after phenol:chloroform extraction of the RNA editing incubation mixtures. The ^{32}P-labelled RNA was ethanol precipitated, dried and dissolved in 5 μl of 50 mM sodium acetate (pH 5.2) containing 0.7 units of P1 nuclease (Sigma). After 1 h of incubation at 37°C, 1–2 μl were loaded on 20 × 20 cm TLC cellulose plates (Merck) and developed with isobutyric acid/NH_3/H_2O (66:33:1 v/v/v) for the first dimension and isopropanol/HCl/H_2O (70:15:15 v/v/v) for the second dimension [14] and autoradiographed.

In RNA editing in vitro assays with the unedited substrate labelled with [^{3}H]- and [^{32}P]RNA, 0.22 pmol (40,000 cpm) of [^{3}H]RNA and 23 fmol of [^{32}P]RNA (24,000 cpm), were mixed and used for editing assays as described above. The cellulose-TLC plates were dried and autoradiographed. The position of the 5′-mononucleotides was located by running unlabelled 5′-NTPs (Sigma) and visualized under UV light. The spots migrating at the positions of pC or pU were scrapped and nucleotides were eluted from cellulose with water. The eluted nucleotides were dropped on nitrocellulose filters, dried and counted in a toluene- based scintillation mixture. The radioactivity of the [^{3}H] channel was corrected for [^{32}P] overlapping which was less than 4%.

2.3. Micrococcal nuclease assay

For micrococcal nuclease (MN) experiments, the mitochondrial lysate was fractionated on MonoQ column (Pharmacia-LKB). The active fractions eluting between 0.10 and 0.15 M NaCl were pooled and concentrated by ammonium sulphate precipitation. Four microliters of the purified fraction was incubated with 1 U of MN (Sigma) in the presence of 1 mM $CaCl_2$. After 2 h incubation at room temperature, the reaction mixture was made 1 mM in EGTA. Four μl of the MN treated proteins were used for the editing reaction in the conditions described [13]. Controls made without MN and without EGTA during

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the editing reaction, or by adding MN + EGTA at the beginning of the RNA editing reaction, were carried out in parallel. After the editing reaction was stopped, the RNA substrate was treated with P1 nuclease as described previously, analyzed by one dimension TLC in isopropanol/HCl/H_2O (70:15:15 v/v/v) and autoradiographed.

3. Results and discussion

Previous studies in our laboratory have shown that mitochondrial extracts of wheat embryos were able to edit faithfully *atp9* mRNA. For this purpose an in vitro assay system using a labelled probed complementary to the edited form of a region of *atp9* mRNA containing 4 editing events was developed [13]. To understand the mechanism of the editing reaction, we used a construction carrying the unedited *atp9* coding region (Fig. 1) to synthesize the RNA substrate labelled at the cytidine residues with α-^{32}P. After incubation with the mitochondrial extract, the RNA was completely digested with P1 nuclease and the 5′-mononucleotides were separated by cellulose thin layer chromatography (TLC) [14]. As shown in Fig. 2b, a fraction of the label was found at the position of UMP (pU) when the substrate was incubated with mitochondrial extract whereas no labelled UMP was obtained when the incubation was done with buffer alone (Fig. 2a). Moreover, no activity was found with heat-inactivated lysate or proteinase K treated extract (not shown). When the editing assay was carried out in the presence of 150 molar excess of tRNA relative to the substrate, no inhibition of the editing activity was observed (Fig. 2c). By contrast, heparin was shown to be a strong inhibitor of the plant mitochondrial RNA editing reaction (Fig. 2d). Heparin is known to interfere with reactions involving an RNA binding step [15]. However, an inhibitory effect of this substance on the enzymatic reaction involved in the C-to-U conversion cannot be excluded. Work is under progress to characterize this RNA binding protein and its mechanism of action in plant mitochondrial RNA editing.

To further characterize the editing reaction, we used shorter unedited RNA substrates: a 66 nucleotides long RNA substrate containing 50 nucleotides corresponding to a region carrying the last four editing sites of unedited wheat *atp9* mRNA (Fig. 1b) and a 25-nucleotide RNA containing only one editing site, the codon 2 of wheat *nad3* mRNA (Fig. 1c). The RNA

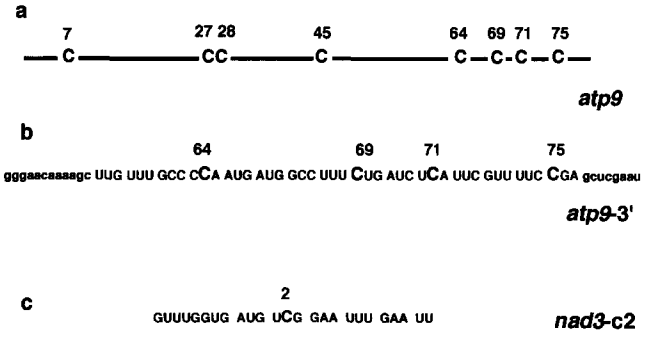

Fig. 1. Substrates used for in vitro RNA editing experiments. (a) Scheme of the 300 nucleotides fragment corresponding to the complete coding region of *atp9*. Only the cytidine residues involved in RNA editing are indicated [13]. (b) Sequence of the short *atp9* RNA fragment (*atp9*-3′) comprising codons 61–75 containing four editing sites. The lowercase letters represent the sequences from the plasmid vector. (c) *nad3* RNA substrate [12]. The bold figures correspond to the codon number of the respective coding regions.

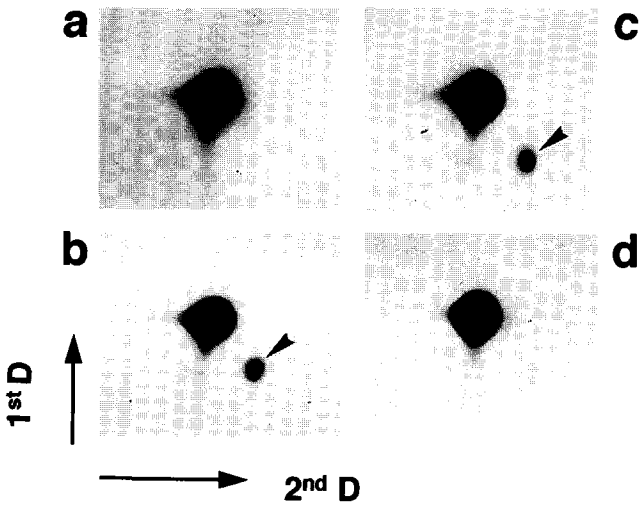

Fig. 2. Two-dimensional cellulose TLC analysis of the edited base. (a) Control reaction without lysate. (b) Incubation with mitochondrial lysate. (c) Editing reaction in the presence of *E. coli* tRNA. (d) Editing reaction performed in the presence of 0.5 μg of heparin. The main spot corresponds to labelled CMP. The black arrow point corresponds to the labelled UMP spot.

substrates were labelled with [α-^{32}P]CTP synthesized by T3 and T7 RNA polymerases, respectively.

Incubation of the [α-^{32}P]RNA with 1, 2 and 4 μg of wheat mitochondrial lysate proteins yielded increasing amounts of a labelled spot migrating at the position of UMP (pU) in a monodimensional TLC (Fig. 3a). Several conclusions can be drawn from this experiment: the substrate used, having a limited amount of coding information, was able to be edited suggesting that the determinants necessary for RNA recognition are located near the editing sites. Thus, long-range interactions and the presence of the whole mRNA seem unnecessary for the faithful RNA editing of plant mitochondrial transcripts. To support this hypothesis we studied the in vitro editing of a shorter mitochondrial RNA fragment. As seen in Fig. 3b, the 26-nucleotide *nad3* fragment carrying one editing site was efficiently edited under the above described conditions. The effect on in vitro RNA editing of deletion and change in the position of the unique editing site of *nad3* are currently under study. In this respect it can be mentioned that a short fragment of *atp9* mRNA located elsewhere of the original gene context, in a chimerical gene transcript, was found to be correctly edited in vivo [16]. We can deduce from these observations that the editing process is not linked to transcriptional or translational events, and that a phosphate molecule remains linked to the modified base.

The involvement of RNA guides in the RNA editing process in trypanosomes [17] has prompted the search of such cofactors in the case of the other editing processes. Our previous preliminary results showed that treatment of the wheat mitochondrial lysate with micrococcal nuclease partially reduced the editing activity [13] suggesting the presence of a nucleic acid factor involved in plant mitochondrial editing. When we addressed this question with the TLC of P1 digestion assay (Fig. 4), we confirmed our previous results that showed the reduced ability of mitochondrial extract treated with MN to carry out the editing reaction. The inhibition observed was in fact due to the effect of EGTA-inactivated nuclease as shown in lane 7 rather

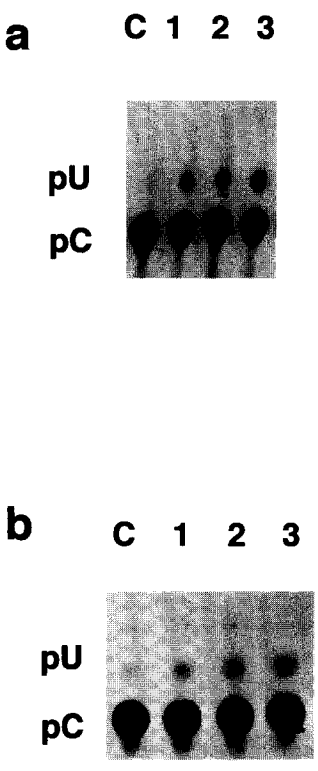

Fig. 3. RNA editing of (a) *atp9* and (b) *nad3* wheat mitochondrial mRNA fragments. ^{32}P-labelled RNA was incubated with mitochondrial extracts and treated with P1 nuclease as described in section 2. One dimension TLC was developed with the mixture: isopropanol/HCl/H_2O (70:15:15 v/v/v). C = Control reaction carried out in the same conditions of the editing assay but in the absence of lysate proteins; 1 = 1 μg; 2 = 2 μg, and 3 = 4 μg of lysate proteins.

than the digestion of a putative RNA contained in the extract. Nuclease inhibition was never complete, and the determination of the A_{280}/A_{260} ratio in active wheat mitochondrial fractions did not indicate the presence of nucleic acids in detectable amounts. These results argue against the involvement of nucleic acids in plant mitochondrial RNA editing. It has been reported that inhibition of enzyme activities by micrococcal nuclease can be explained by a substrate masking effect [17]. A similar result has been observed in the apolipoprotein B editing system [18]. In the latter case, MN inhibition has been explained by the generation of inhibitors after nuclease treatment of crude extracts. Moreover, we have observed that the editing activity was still present after several chromatographic steps that should have deprived the active fractions of nucleic acids. Although these observations cast some doubts about the participation of free RNA molecules in the editing reaction in plant mitochondria, the possibility that such molecules can be strongly bound to the functional proteins and thus inaccessible, cannot be excluded. The apparent stimulation effect of Ca^{2+} (lane 3) seems to be not significant since it was not observed in further studies and was probably due to the partial digestion of RNA with P1 nuclease.

To understand the mechanism of RNA editing in plant mitochondria, the unedited RNA probe was incubated with the lysate prepared as in [13] or with fractions obtained by chromatography on MonoQ column. After one or two hour incubation, no digestion of the RNA probe was observed, suggesting that no endonucleolytic activity is detected in the extract (results not shown). An endonucleolytic activity would be required by a nucleotide replacement mechanism [19]. Two other biochemical pathways may explain a C-to-U conversion: base exchange and base modification (Fig. 5).

Two dimensional TLC analysis of the [α-^{32}P]-labelled RNA substrate, digested with P1 nuclease after the editing reaction, showed that the label of pC is found in the nucleotide migrating at the position of genuine pU (Fig. 2). This result clearly show that the ribose-phosphate moiety is not affected during the RNA editing process. A similar observation was made in organello by Rajasenkar and Mulligan [20]. Thus, a cytosine modification such as a deamination or a transglycosylation can be postulated as involved in the C-to-U conversion in plant mitochondria. A deamination mechanism has been proposed to explain the unique C-to-U conversion of apolipoprotein B mRNA in mammals [21,22] a well as the A-to-I change in glutamate-gated ion channel in brain [23].

A more convincing evidence of C-to-U conversion through a deamination mechanism was obtained by performing the editing reaction with a mixture of unedited *atp9* mRNA fragments, labelled with ^{3}H on the base and with ^{32}P in the α-phosphate of the C residue, respectively (Fig. 6). The RNA was incubated with the mitochondrial lysate as described above. After complete P1 nuclease digestion, analysis of pC and pU spots showed co-migration of the ^{3}H and ^{32}P label at the position of UMP. The ^{3}H/^{32}P radioactivity ratio, determined in

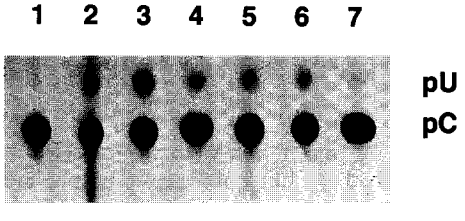

Fig. 4. Micrococcal nuclease effect on RNA editing activity. Labelled substrate (*atp9*-3′) was incubated with MonoQ active fractions (F1) pre-incubated with micrococcal nuclease. The products were analysed by one dimension TLC developed with the mixture: isopropanol/HCl/H_2O (70:15:15 v/v/v). 1 = RNA substrate alone; 2 = F1 without MN treatment; 3 = F1 preincubated with Ca^{2+}; 4 = F1 preincubated with Ca^{2+} and EGTA; 5 = F1 preincubated with 1 U MN; 6 = F1 preincubated with 2 U MN; 7 = F1 preincubated with Ca^{2+} alone and then 2 U of MN plus EGTA were added during the editing reaction. For details see section 2.

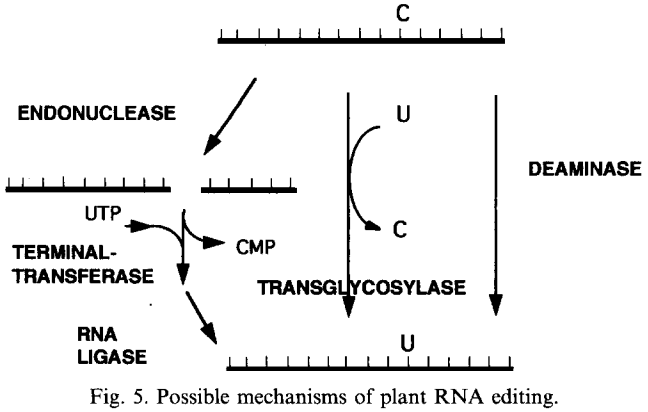

Fig. 5. Possible mechanisms of plant RNA editing.

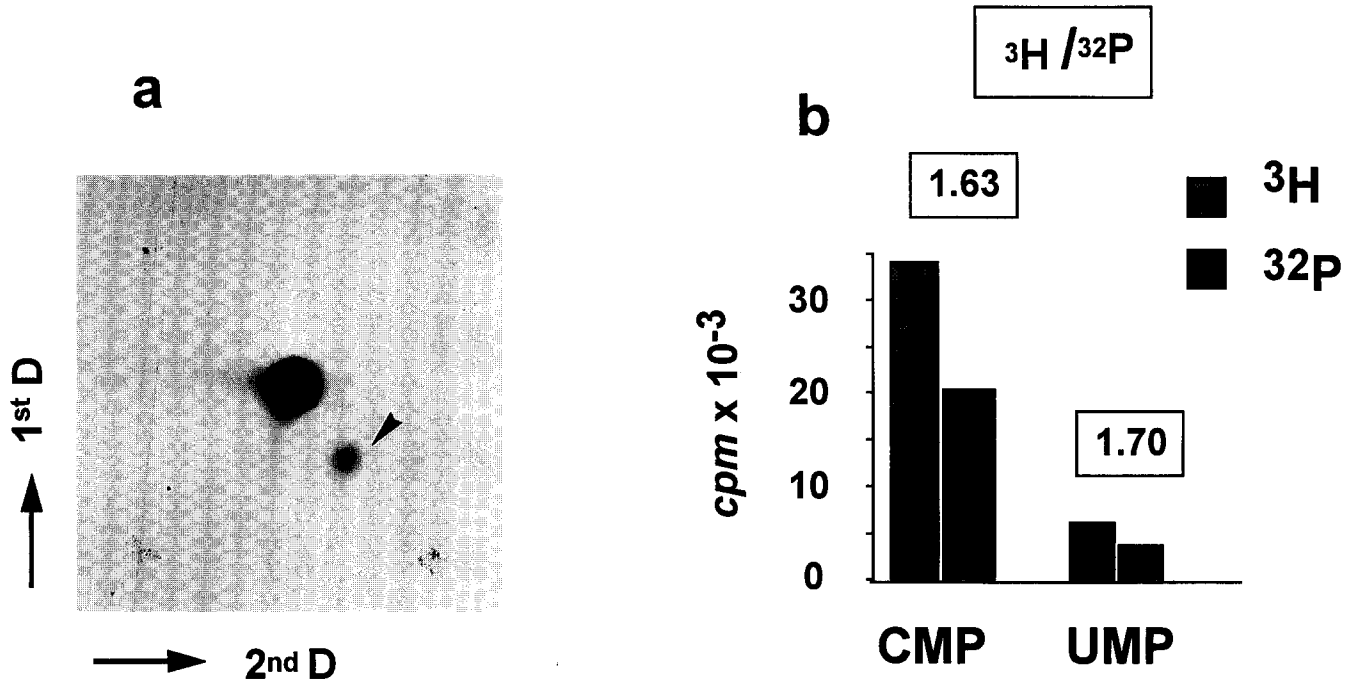

Fig. 6. Double dimension analysis of *atp9* RNA labelled with ^{3}H and ^{32}P after incubation with wheat mitochondrial lysate. (a) After incubation with mitochondrial proteins, the $^{3}H/^{32}P$-labelled RNA was submitted to P1 digestion and the mononucleotides were separated by 2D cellulose-TLC. After chromatography, the TLC plate was autoradiographed. Spots at the position of pC and pU were eluted and the radioactivity dertermined as described in section 2. (b) Histogram of the radioactivity, corrected for background and for ^{32}P to ^{3}H overlapping, obtained by counting the CMP and UMP spots. The $^{3}H/^{32}P$-ratios are boxed.

either the pC or the pU spots after TLC, was very similar and are in good agreement with the original ratio of these isotopes in the labelled *atp9* mRNA used in the assay. These results strongly suggest that the conversion of the cytosine into a uridine residue indeed operates through a deamination mechanism and exclude a transglycosylation step. In fact, the last mechanism should have led to the lost of the ^{3}H-label in the pU spot.

The results presented in this paper suggest that the editing process in plant mitochondria can occur on short substrates outside of their original context. The examination of many plant mitochondrial RNA sequence sites showed that no consensus motifs are present capable to explain the specificity of the reaction. The presence of guide RNA (gRNA) molecules as determinants of specificity has been suggested (W. Schuster, personal communication). We have no conclusive evidence for the participation of gRNA in our system, as suggested by the micrococcal nuclease experiment (see above), but this possibility deserves investigation. Further studies of 'unedited RNA' binding proteins should help to clarify this point.

Our results provide compelling evidence that in plant mitochondria RNA editing proceeds by a deamination mechanism. A cytidine deaminase activity has been detected when a cloned gene involved in apolipoprotein B mRNA editing is expressed in *Xenopus* oocytes [24]. In contrast with this result, we have not detected such activity in active wheat mitochondrial extracts.

In chloroplast some transcripts are also edited by a C-to-U transition. It should be interesting to study if both organellar systems share some protein factors to fulfill the editing process.

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References

[1] Hodges, P. and Scott, J. (1992) Trends Biochem. Sci. 17, 77–81.
[2] Sommer, B., Köhler, M., Sprenzel, R. and Seeburg, P.H. (1991) Cell 67, 11–19.
[3] Gualberto, J.M., Lamattina, L., Bonnard, G., Weil, J.H. and Grienenberger, J.M. (1989) Nature 341, 660–662.
[4] Covello, P.S. and Gray, M.W. (1989) Nature 341, 662–666.
[5] Hiesel, R., Wissinger, B., Schuster, W. and Brennicke, A. (1989) Science 246, 1632–1634.
[6] Hernould, M., Suharsonno, S., Litvak, S., Araya, A. and Mouras, A. (1993) Proc. Natl. Acad. Sci. USA 90, 2370–2374.
[7] Covello, P.S. and Gray, M.W. (1993) Trends Genet. 9, 265–268.
[8] Hiesel, R., Combettes, B. and Brennicke, A. (1994) Proc. Natl. Acad. Sci. USA 91, 629–633.
[9] Araya, A., Bégu, D. and Litvak, S. (1994) Physiol. Plant. 91, 543–550.
[10] Bégu, D., Graves, P.V., Domec, C., Arselin, G., Litvak, S. and Araya, A. (1991) Plant Cell 2, 1283–1290.
[11] Hoch, B., Maier, R.M., Appel, K., Igloi, G.L. and Kössel, H. (1991) Nature 353, 178–180.
[12] Gualberto, J.M., Bonnard, G., Lamattina, L. and Grienenberger, J.M. (1991) Plant Cell 3, 1109–1120.
[13] Araya, A., Domec, C., Bégu, D. and Litvak, S. (1992) Proc. Natl. Acad. Sci. USA 89, 1040–1044.

[14] Nishimura, S. (1972) Progr. Nucleic Acid Res. Mol. Biol. 12, 49–85.
[15] Konarska, M.M. (1989) Methods Enzymol. 180, 442–453.
[16] Kumar, R. and Levings III, C.S. (1993) Curr. Genet. 23, 154–159.
[17] Blum, B., Bakalara, N. and Simpson, L. (1990) Cell 60, 189–198.
[18] Wang, M.J. and Gegenheimer, P. (1990) Nucleic Acids Res. 18, 6625–6631.
[19] Greeve, J., Navaratnam, N. and Scott, J. (1991) Nucleic Acids Res. 19, 3569–3576.
[20] Rajasenkar, V.K. and Mulligan, R.M. (1993) Plant Cell 5, 1843–1852.
[21] Navaratnam, N., Shah, R., Patel, D., Fay, V. and Scott, J. (1993) Proc. Natl. Acad. Sci. USA 90, 222–226.
[22] Harris, S.G., Sabio, I., Mayer, E., Steinberg, M.F., Backus, J.W., Sparks, J., Sparks, C.E. and Smith, H.C. (1993) J. Biol. Chem. 268, 7382–7392.
[23] Yang, J.-H., Sklar, P., Axel, R. and Maniatis, T. (1995) Nature 374, 77–81.
[24] Navaratnam, N., Morrison, J.R., Bhattacharya, S., Patel, D., Funahashi, T., Giannoni, F., Teng, B., Davidson, N.O. and Scott, J. (1993) J. Biol. Chem. 268, 20709–20712.